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Polypeptide and Polysaccharide Processing in Hyperthermophilic Microorganisms

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FINAL REPORT

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R.M. Kelly

Department of Chemical and Biomolecular Engineering
North Carolina State University
Raleigh, NC 27695-7905

Executive Summary: This project focused on the microbial physiology and biochemistry of heterotrophic hyperthermophiles with respect to mechanisms by which these organisms process polypeptides and polysaccharides under normal and stressed conditions. Emphasis is on two model organisms, for which completed genome sequences are available: *Pyrococcus furiosus* (growth T_{opt} of 98°C), an archaeon, and *Thermotoga maritima* (growth T_{opt} of 80°C), a bacterium. Both organisms are obligately anaerobic heterotrophs that reduce sulfur facultatively. Whole genome cDNA spotted microarrays were used to follow transcriptional response to a variety of environmental conditions in order to identify genes encoding proteins involved in the acquisition, synthesis, processing and utilization of polypeptides and polysaccharides. This project provided new insights into the physiological aspects of hyperthermophiles as these relate to microbial biochemistry and biological function in high temperature habitats. The capacity of these microorganisms to produce biohydrogen from renewable feedstocks makes them important for future efforts to develop biofuels. A summary of significant accomplishments in focus areas is provided in the following sections.

- (1) Development of whole genome spotted cDNA microarrays in conjunction with experimental and statistical methodologies for *P. furiosus* and *T. maritima* studies**
- (2) Genome-wide transcriptional response of *T. maritima* and *P. furiosus* to carbohydrate carbon/energy sources**
- (3) Exopolysaccharide/biofilm formation by *T. maritima***
- (4) Thermal stress response by *P. furiosus* and *T. maritima***
- (5) Role of 20S proteasome β_1 protein in *P. furiosus* during thermal stress**
- (6) Response of wild-type *T. maritima* and a resistant mutant to antibiotic challenge**
- (7) Response of *T. maritima* to different acetate levels in the growth medium**
- (8) Physiological/biochemical characterization of proteases from *P. furiosus* and *T. maritima***

(1) Development of whole genome spotted cDNA microarrays and experimental and statistical methodologies for *P. furiosus* and *T. maritima* studies

- Designed, printed and implemented targeted and whole genome cDNA microarrays (based on 300-500 bp PCR products) for *P. furiosus* (~2,000 ORFs) and *T. maritima* (1878 ORFs) in our laboratory. Also, through a collaboration with SAS Institute (Cary, NC), developed and implemented data analysis tools, using a mixed effects statistical model, for transcriptional response experiments (e.g., see Chhabra et al., 2003).
- Developed batch and continuous culture protocols and capabilities for *P. furiosus* and *T. maritima* for high temperature, anaerobic growth in conjunction with appropriate sampling methodologies to ensure meaningful transcriptional response information.
- Evaluated the influence of biological variability as this relates to differential gene expression. Showed for maltose-limited, continuous growth of the hyperthermophile *T. maritima* at different dilution rates and temperatures ($80^{\circ}\text{C}/0.25\text{ h}^{-1}$, $80^{\circ}\text{C}/0.17\text{ h}^{-1}$, and $85^{\circ}\text{C}/0.25\text{ h}^{-1}$) that transcriptome-wide variation in gene expression within mechanical steady states was minimal compared to between steady states, supporting the efficacy of chemostat-based approaches for functional genomics studies (Shockley et al., 2005). Similar results were obtained for *P. furiosus* maltose- and cellobiose-limited chemostat cultures (Chou et al., in preparation). Also, examined differential gene expression as a function of growth phase for batch cultivation of *T. maritima* (Johnson et al., 2006) and *P. furiosus* (Chou et al., 2007)

(2) Genome-wide transcriptional response to carbohydrate carbon/energy sources

- Examined *T. maritima* response to growth on 14 different carbohydrates with the objective of assigning ABC transporters to specific sugar substrates and associated transcriptional regulators. Showed that simple changes in carbohydrate energy/carbon source can have profound effect on genome-wide gene expression and gene regulation (Chhabra et al., 2003; Connors et al., 2005). Roles in uptake of specific carbohydrates were suggested for members of the expanded Opp/Dpp family of ABC transporters. In this family, phylogenetic relationships among transport systems revealed patterns of possible duplication and divergence as a strategy for evolution of new uptake capabilities.
- Examined *P. furiosus* response to growth on 6 different glucans with the objective of identifying carbon utilization patterns (Lee et al., 2006). Also, showed that substantial differences in gene expression patterns result when sulfur is added to maltose-grown cultures compared to cellobiose-grown cultures (Chou et al., 2007).
- Showed that the effect of sulfur on *T. maritima* growth on carbohydrates (maltose, cellobiose) is significantly different from *P. furiosus*.

(3) Exopolysaccharide/biofilm formation by *T. maritima*

Used chemostat culture to obtain samples of biofilm-bound *T. maritima* for transcriptional response analysis. In addition to comparing sessile and planktonic phenotypes, showed that significant up-regulation of β -specific glycosidases in biofilm-bound cells likely relates to hydrolysis of exopolysaccharide matrix of biofilm (Pysz et al., 2004; Johnson et al., 2006).

(4) Thermal stress response by *P. furiosus* and *T. maritima*

- Dynamic transcriptional profiles for thermal stress response were obtained from growth experiments conducted in a 14-liter, high temperature fermenter for *T. maritima* (80 → 90°C) and *P. furiosus* (90 → 105°C) using targeted (Shockley et al., 2003; Pysz et al., 2004) and whole genome (Shockley, 2004) cDNA microarrays.
- In *T. maritima*, early induction of predicted heat shock operons *hrcA-grpE-dnaJ* (TM0851-TM0850-TM0849), *groES-groEL* (TM0505-TM0506), and *dnaK-sHSP* (TM0373-TM0374) was consistent with conserved CIRCE elements upstream of *hrcA* and *groES*. Induction of the *T. maritima* *rpoE/sigW* and *rpoD/sigA* homologs suggests a mechanism for global heat shock response in the absence of an identifiable ortholog to a major heat shock sigma factor. Notably, *T. maritima* showed indications of a late heat shock response with the induction of a *marR* homolog (TM0816), several other putative transcriptional regulators (TM1023, TM1069), and two closely related α -glucuronidases (TM0434 and TM1068). While *T. maritima* shares core elements of the bacterial heat shock response with mesophiles, the thermal stress regulatory strategies of this organism differ significantly (Pysz et al., 2004).
- Differential gene expression analysis of *P. furiosus* response to heat shock reveals a cooperative strategy of rescue (thermosome (Hsp60), small heat shock protein (Hsp20), two VAT-related chaperones), proteolysis (proteasome) and stabilization (compatible solute formation) to cope with polypeptide processing during thermal stress (Shockley et al., 2003).
- Despite their grouping with different domains of life, dynamic response to thermal stress for *T. maritima* and *P. furiosus* shows common regulatory features (Shockley, 2004).

(5) Role of 20S proteasome β_1 protein in the hyperthermophilic archaeon *Pyrococcus furiosus* during thermal stress

- Transcriptional analysis of *P. furiosus* subjected to heat shock (shift from 90 to 105°C) showed that expression of the β_2 gene (PF0159) in the archaeal proteasome was affected differently than the β_1 gene (PF1404), suggesting different roles in thermal stress response for the subunits (Madding et al., 2007).
- Recombinant versions of individual proteins varied significantly in thermostability, as evidenced by melting temperatures of 104°C, and 93°C for β_1 , and β_2 , respectively. Consistent with transcriptional data, the β_1 subunit was incorporated into the 20S proteasome at 105°C more so than at 90°C.
- The $\alpha+\beta_1+\beta_2$ assembly at 105°C was significantly more thermostable than other assemblies, based on its melting behavior and seven-fold lower inactivation rate upon incubation at 115°C.
- These results indicate that the β_1 protein in the *P. furiosus* 20S proteasome plays a thermostabilizing role at supraoptimal temperatures and suggests that subunit composition can be a factor in proteasome function during thermal stress when polypeptide turnover is essential to cell survival (Madding et al., 2007).

(6) Response of wild-type *T. maritima* and a resistant mutant to antibiotic challenge

- Whole-genome dynamic transcriptional response of wild-type *T. maritima* and a resistant mutant were followed for exposure to chloramphenicol (CAM) in both batch and continuous culture (Montero et al., 2007).
- Significant differences were observed between the phenotypes of the wild-type and mutant before and after antibiotic challenge. The resistant mutant was pre-conditioned to (CAM) challenge as evidenced by increased synthesis of ribosomal DNA and polyamines compared to the wild-type strain in the absence of CAM. Under CAM challenge, the mutant exhibited similar behavior to the wild-type, albeit requiring longer antibiotic exposure times to develop.
- As expected for bacteria responding to translation inhibitors (such as CAM), transcriptional response of the wild-type *T. maritima* strain showed a general down-regulation of macromolecular synthesis. This included biosynthesis of amino acids (in particular, Met, His, Val, Leu, Ile, Gly, Lys, Thr, Ser, Glu, Try, Cys), purines, structural components of the F1/F0 ATP synthase, and the cellobiose uptake transporter (main carbon/energy source in this experiment).
- In both cases, increased synthesis of the translational apparatus was observed, including several ribosomal proteins, transcriptional factors (e.g., IF-3, greA, Ef-Tu, NusA). Also up-regulated were: transcriptional regulators (e.g., Sigma A, Sigma E and oxidative stress proteins), cold shock proteins and protein chaperones involved in heat shock response, flagella genes and methyl-accepting proteins (suggesting a negative chemo-tactic response), oxidative stress proteins (e.g., ferredoxin, ferritin, dehydrogenases and pyridoxine synthesis), the SRP-secretion pathway (suggesting an antibiotic-induced selection of co-translational translocation over the general secretory pathway), polyamine biosynthetic pathway, and components of the trans-translation (tmRNA) pathway.

(7) Response of *T. maritima* to different acetate levels in the growth medium

- Analyzed the whole genome transcriptional response of *T. maritima* to an acetate pulse addition (150 mM final acetate concentration) during mid-exponential batch growth in a 14-liter bioreactor. *T. maritima* demonstrated a decreased growth rate and a stress response similar that shown under thermal shock.
- Transcriptional profiles were developed for *T. maritima* grown with 2-liter bioreactors at pH 5.6 and pH 6.8 conditions in growth media with and without 40mM acetate. Analysis showed a differential cellular response to acetate at pH 5.6 compared to pH 6.8, specifically the heat shock protease HsIV (TM0522) and ATP-dependent Zn protease (TM0580) showed a significant up-regulation to acetate only under low pH, high acetate conditions.
- The reduction of media pH for *T. maritima* cells growing in the presence of acetate yielded a transcriptional response for only proteolytic genes associated with shock response.
- Whole genome cDNA microarrays in combination with experimental and statistical methods developed in house facilitated the ability to distinguish between transcriptional changes due to pH and extracellular acetate.

(8) Physiological/biochemical characterization of proteases from *P. furiosus*/*T. maritima*

- Continued efforts to study the biochemical and biophysical features of a range of proteases from *P. furiosus* and *T. maritima*. Cloned and expressed active forms of several protease/peptidase-related genes in *Escherichia coli*, including several *T. maritima* and *P. furiosus* peptidases that are related to TET peptidase in *Haloarcula marismortui*.
- Cloned and expressed several proteins possibly associated with the cellular membrane: periplasmic heat-shock serine protease (TM0571), periplasmic serine protease (TM0916), and Clostripain-related protein (TM1589).
- Cloned, expressed, and biochemically characterized an oligopeptidase (TM0963), PepF, from *T. maritima* that demonstrates a high cleavage specificity for small peptides with a Arg-Arg or Phe-X amino acid sequence.
- Continued work on the cloning and expression of proteins from *T. maritima* (TM0785), *P. furiosus* (PF1191), and *S. solfataricus* (SSO2749) that are related to a *Brevibacterium linens* bacteriocin using recombinant *E. coli* and *Pichia pastoris* cloning systems.
- Native purification continues for the bacteriocin-related proteins directly from *T. maritima* and *P. furiosus* high cell-density cultures. Optimal conditions for induction of TM0785 and PF1191 were deduced from cDNA microarray experiments, which showed a high transcriptional dependence on growth phase and growth rate.

Publications acknowledging DOE support from this Project Period:

Refereed journal articles published:

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Students supported in part by this grant during the period covered in progress report:
(initial positions of students upon leaving the Kelly Lab are indicated)

Swapnil Chhabra	PhD, 2003	Postdoc, Sandia National Lab
Lara Chang	PhD, 2003	Postdoc, Dept. of Pathology, Duke Medical Center
Marybeth Pysz	PhD, 2004	Postdoc, Roswell Cancer Center, Buffalo, NY
Keith Shockley	PhD, 2004	Postdoc at the Jackson Laboratory, Bar Harbor, ME
Shannon Connors	PhD, 2005	Bioinformaticist, SAS Institute, Cary, NC
Matthew Johnson	PhD, 2005	Research Engineer, Wyeth, RTP, NC
Joshua Michel	PhD, 2007	Research Engineer, Genencor, Palo Alto, CA
Jason Nichols	Postdoc (2004-07)	Research Scientist, Syngenta, RTP, NC
Amy Vanfossen	PhD student	Will complete PhD in 2009
Steven Gray	PhD student	Will complete PhD in 2009